Spinning Disk Microscope

- User's Guide -

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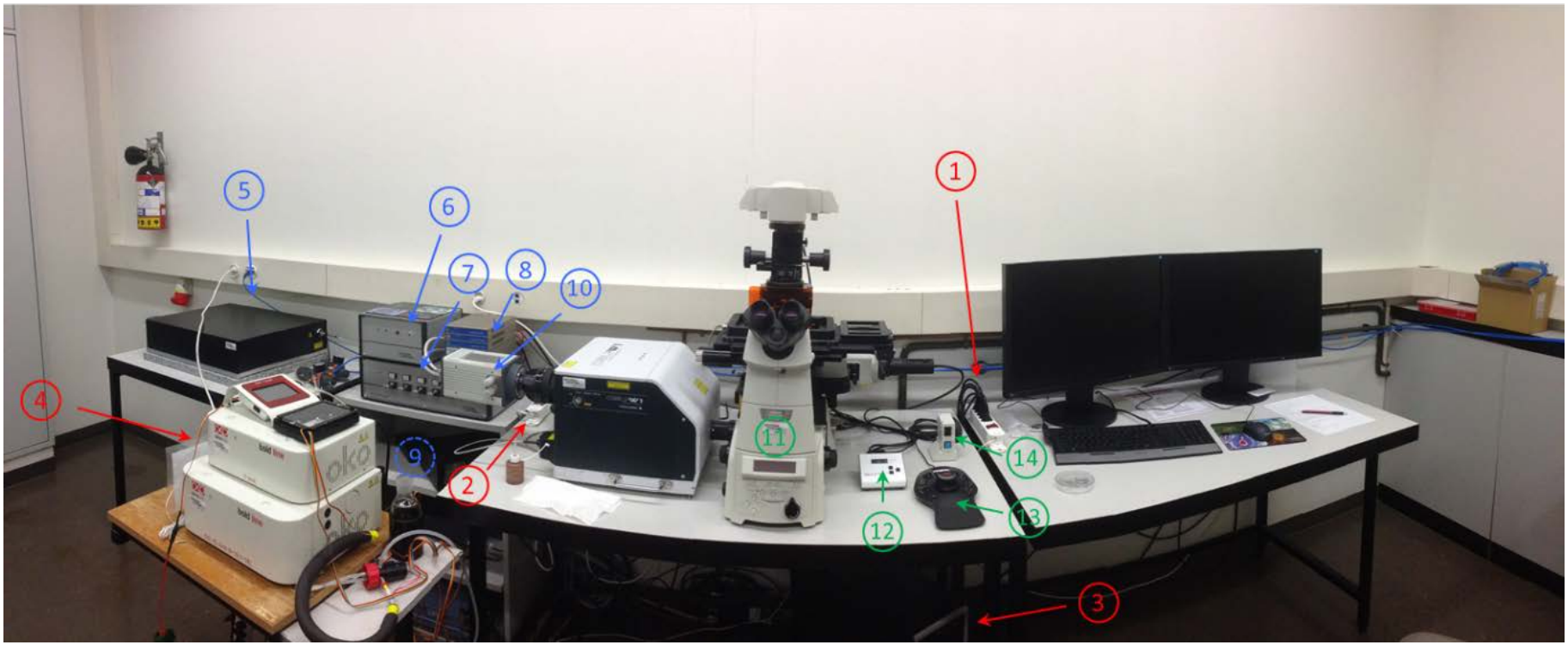


COMPONENTS

Red = Hardware that needs to be operated

Blue = Hardware that normally does not need to be operated

Green = Microscope components

1. Power Switch (Scope, Stage, Lasers)
2. Power Switch (LED, Confocal, Camera)
3. Computer
4. Incubator
5. Stage top Laser mixer
6. Optical modulator
7. Laser controller with switches
8. Stage controller
9. Fluorescent LED array
10. EMCCD camera
11. Microscope
12. Bright field lamp controller
13. X,Y stage joystick
14. PFS controller/offset controller

TURNING ON SEQUENCE

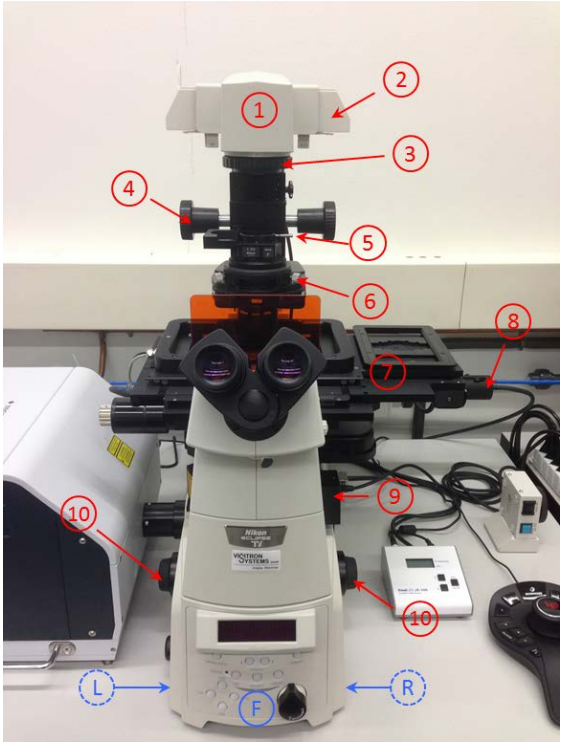
All the equipment is connected to the power switches **1** and **2**.

! Please follow the order, otherwise the Epifluorescence light may not work.

1) Switch ON all microscope components (**1** and **2**)
2) Turn ON Computer (**3**)
3) If needed, switch ON Incubator (**4**). Please report if the fuse goes off!
4) Turn ON laser keys (**7**) (usually they are already ON by default).
5) Log in to the Computer with your ZMB user account.
6) Start the VisiView Software

MICROSCOPE PARTS

1. Bright field source
2. Filters (normally no need to manipulate them)
3. Field aperture diaphragm
4. Condenser height controller
5. DIC polarizer (push lever to left to enable DIC)
6. Condenser (motorized via software)
7. X,Y stage
8. Fluorescent light source input
9. emission filter cube (not to be touched)
10. Z-Focus Wheel

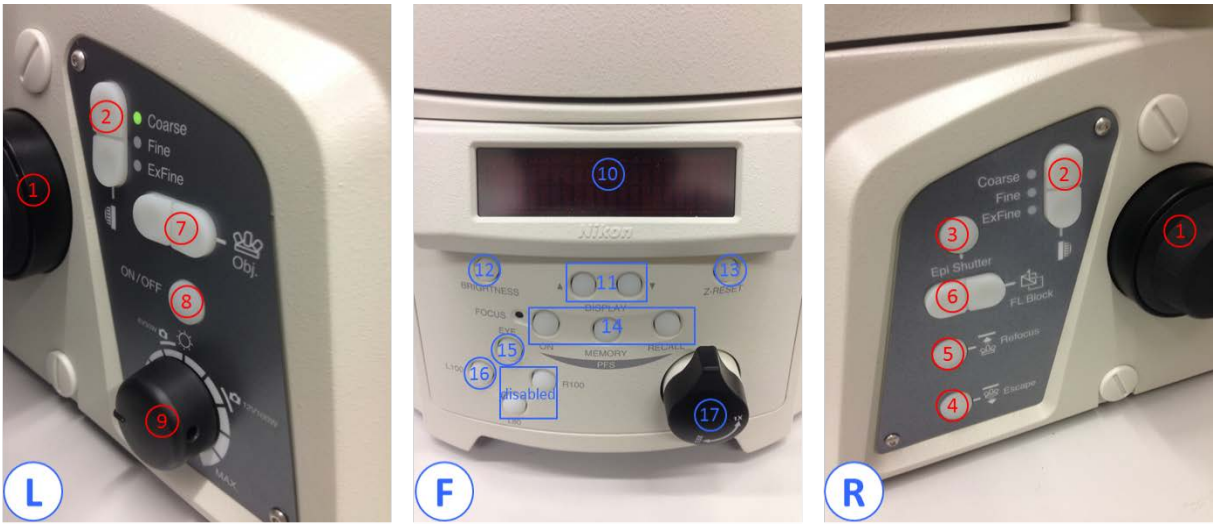


The X,Y stage (**7**) is controlled with the joystick (**13, front page**). It can be moved in any X,Y direction.

- Coarse mode movement – shift joystick
- Fine mode movement – tilt joystick

Microscope Panels (L = left; F = front; R = right)

1. Z joystick (focus)
2. Z mode selection (coarse, fine, extra fine)
3. Epifluorescence Shutter (disabled)
4. Escape button
5. Refocus button
6. Emission filter block switcher
7. Objective selection switcher
8. Brightfield lamp switch (disabled)
9. Lamp intensity (disabled)
10. Display
11. Display info selection
12. Display brightness
13. Z value reset
14. PFS controllers
15. Eyepiece view
16. Camera view
17. Additional 1.5x Zoom lens (not recommended, not recognized by software)



The position of the Z focus is displayed on the front screen (F**10**). This value can be reset to 0 at any point (**13**) if this helps to localize a specific working plane (only recommended if all the samples are in the same plane or a highly similar one).

The type of information displayed on the screen can be changed to any that suits best (F**11**).

The brightness of the display can also be modified (F**12**).

- Escape button (R**4)** - objective moves down (and the focus wheel is temporarily disabled)
- Refocus (R**5**) – goes back to focus position (does not have to be set, as it will be remembered)

The images can be directed to the eyepiece or to the camera using the buttons in the front panel (F**15** and F**16** respectively). The configuration might not always change automatically from eyepiece to camera when the software is commanded to acquire, so make sure you have pressed the according button.

Zoom 1.5 lens:

The zoom (F**17**) is only useful if you are working with slides and few focal planes. Moreover, the software does not recognize it, and thus the scale bars are not appropriately adjusted. Make sure that it is in the 1x position before starting acquisition.

OBJECTIVES

Name	*Magnification*	*NA*	*Immersion*	*WD (mm)*	*Pinhole diameter (um)*
CFI PLANAPOCHROMAT DIC	10x	0.45	Dry	4.0	25
CFI S PLANFLUOR ELWD Ph	20x	0.45	Dry	6.9-8.2	25
CFI S PLANFLUOR ELWD Ph	40x	0.6	Dry	2.8-3.6	25
CFI PLANFLUOR DIC	20x	0.75	Water, Glycerol, Oil	0.33-0.35	25
CFI PLANAPOCHROMAT VC DIC	60x	1.4	Oil	0.13	50
CFI PLANAPOCHROMAT DIC	100x	1.4	Oil	0.13	50

20x and 40x dry = long working distance objectives. They have a collar ring to adjust the thickness of the cover glass. Make certain that you adjust it properly, image quality can suffer otherwise.

The objectives can be changed either from the software (recommended) or manually. If they are changed via software, the revolver escapes down, the objective is changed to the one requested and the revolver goes back to the previous focus position. The same happens if the objectives are changed with the selection button (L**7**) at the microscope stand, but the escaping/refocusing cycle takes place between every objective, not only the one of our interest. To avoid this and move faster, the escape button (R**4**) can be pressed, then the objective is selected (L**7**) and finally refocus by pressing the button (R**5**). Escape (R**4**) and refocus (R**5**) buttons can also be used to exchange samples, add more immersion media, etc.

Note: Toggling between objectives directly at the microscope, will not be recognized by the software – It is recommended to toggle between objectives only with the help of the software

PERFECT FOCUS SYSTEM CONTROLLER

The **Perfect Focus System (PFS)** automatically maintains microscope focus so that the point of interest within a specimen is always kept in sharp focus, no matter what mechanical or thermal changes take place. It never focuses on the sample; it recognizes the closest interface between different materials (for example between glass coverslip and sample) and follows it, no matter if there are changes in the Z plane. It is a very useful tool for live cells.

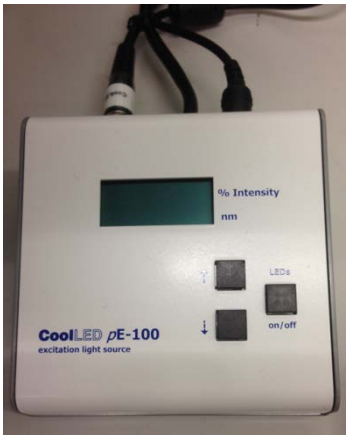
To activate it, focus your sample normally and then turn on the PFS controller (right picture). Immediately, the left-most button in the front panel of the microscope (**F14**) turns green. If it is blinking, it means it is ON but cannot detect a valid interface and has not engaged. Moving the Z focus up and down until a valid interface is detected will cause it to engage. If it is green (without blinking), it is ON and engaged.

- Blinking – looking/searching for interface
- Solid ON – found a focus point

Once activated, the Z focus controls at the microscope (**L1, L2, R1, R2** - see pages 4 and 5**)** are disabled. The focal plane can then only be modified/adjusted with the PFS controller wheel/offset controller. Coarse or fine modes are selected with the blue button.

NOTE: The PFS can interfere with the acquisition of Z-stacks as it will start racing after the piezo stage if active during Z-stack acquisition. Make sure that the PFS is manually turned off during Z-stack acquisition or that the "Autofocus on during waiting" option is checked in the "Time Lapse" tab of the "Acquire" dialog (See page 14). This option will ensure that the PFS is turned on and off automatically by the software as needed.

BRIGHT FIELD CONTROLLER



Use the bright field controller (**12**, front page) to turn ON the source or modify the light intensity (arrow up or down buttons).

For brightfield/transmitted light a cooled green LED (525nm) is used. This is intentioned to avoid photo bleaching of the sample while focusing.

This Led is only controllable by the panel and NOT via the software.

The BF source turns OFF automatically during image acquisition.

NOTE: If fluorescence is not visible in the screen, check first that the BF source is off.

However, for bright field/transmitted light images, anytime a new objective is used, **Köhler illumination** should be adjusted:

1) close the aperture (**3,** page 4) until you can see a polygon in the screen
2) move the two alignment screws until the polygon is centered
3) move the condenser position (**4,** page 4) until the edges of the polygon are sharp
4) open the aperture (**3**, page 4) again until the whole field is covered with light.

NOTE: If the polygon is not visible it could be totally out of alignment and out of view. This can happen with high magnification objectives and in that case try the procedure with a lower magnification first and then move back to the higher magnification objective and repeat.

TURNING OFF SEQUENCE

Check on the reservation system whether another user has registered for the spinning disk right after you.

If YES:

1) Make sure your pictures are saved in your folder on drive H
2) Close the software. There is no need of turning ON/OFF lasers in the software.
3) Clean the used lenses
4) Log OFF

If NO:

1) Make sure your pictures are saved in your folder on drive H
2) Close the software. There is no need of turning ON/OFF lasers in the software.
3) Clean the used lenses.
4) Shut down the computer
5) Switch OFF switches **1** and **2**
6) Switch OFF incubator, if it has been used (the order is not important)
7) Cover the microscope

VisiView® imaging software

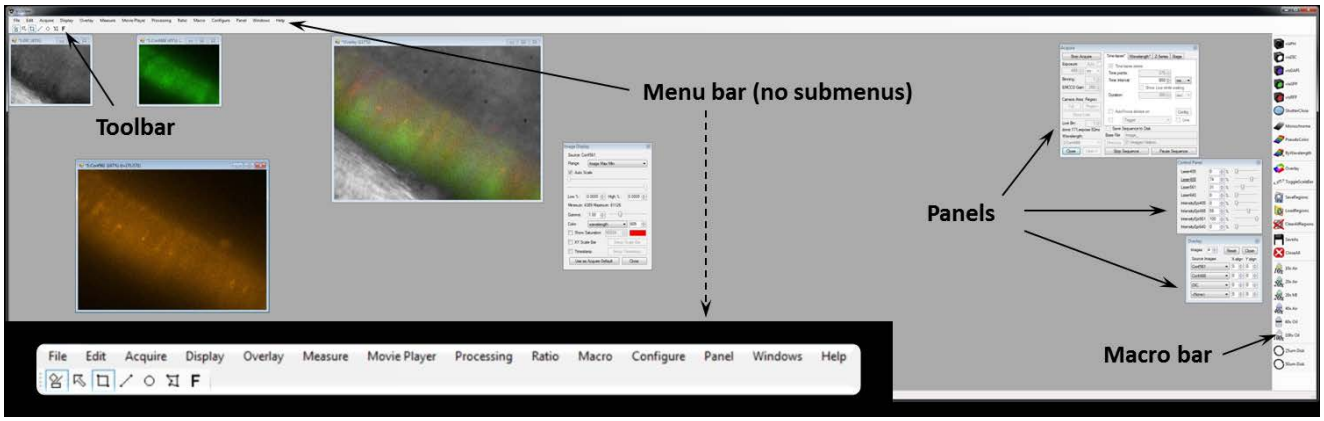
VisiView® opens on both computer screens and is macro based. This means that many functions are freely programmable.

NOTE: Software needs to always be turned ON, even if only the microscope will be used.

While the software is starting you will hear a “clack”. This is normal and comes from the Piezo stage which goes to its middle position at 175um (full travel range = 350um).

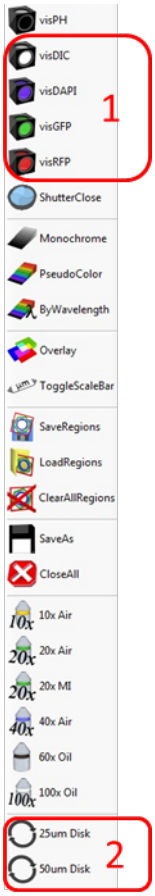
NOTE: For reasons of simplicity, there are no submenus in the main menu bar: Some menus open to reveal a simple list of menu items and others, when chosen, simply open a panel. (If this is not the case, the panel is already open and minimized somewhere).

The software opens up as follows (without the fluorescence images):


Toolbar
Menu bar (no submenus)
Panels
Macro bar
File Edit Acquire Display Overlay Measure Movie Player Processing Ratio Macro Configure Panel Windows Help

MACROS

Tabs on right side of the screen = Macros



The **macro bar** contains some of the most used functions.
From here, the fluorescence lamp source is controlled (**1**).

NOTE: Fluorescence only controllable via Software – to view sample directly at the microscope the appropriate fluorescence filter has to be chosen and click "Show Live" (see acquire panel below)

The pinhole diameter for confocal imaging can also be chosen for each objective (**2**).

2 spinning disks are available: 25um pinholes (air 10, 20 and 40x and 20x MI)
50um pinholes (oil 60x and 100x)

NOTE: After selecting the objective, make sure that you also have chosen the corresponding pinhole disk (see above).

To run a macro directly, click on it.

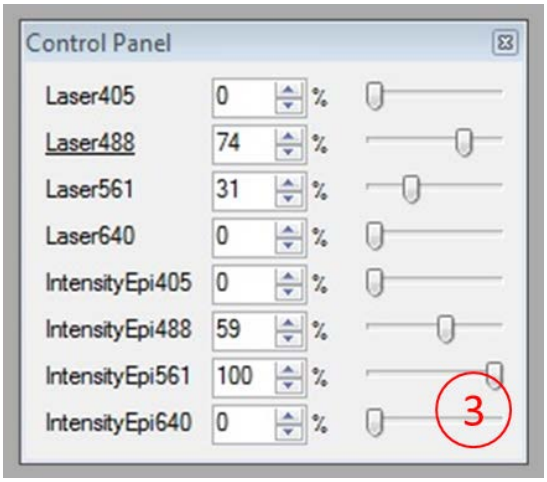
Any **macro** can be modified to, for instance, create a work pipeline. However, this requires some programming skills.

Edit Macro: "Shift" + left mouse button on particular macro icon

NOTE: This is global – every change will be recognized by every user! Therefore it is IMPORTANT that you create your own. DO NOT change any of the existing macros; always say "Save As…". You can always add your own macros in the macro bar while removing the default ones. The macro bar is personalized for each user.

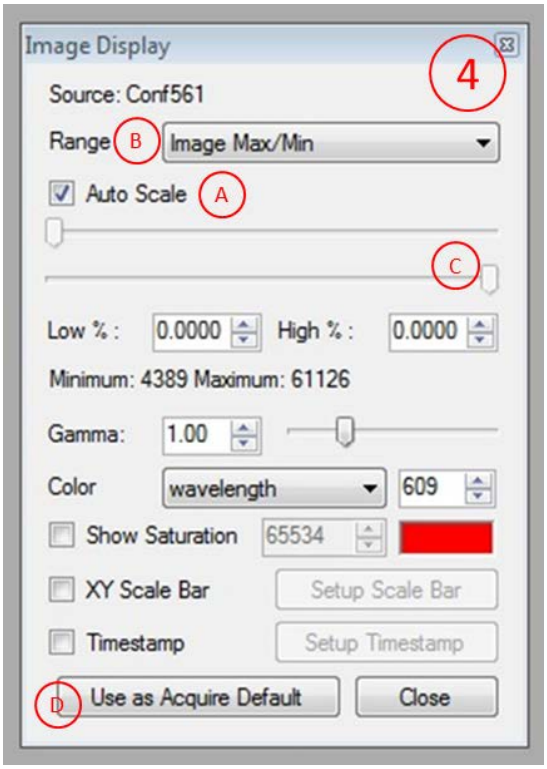
Click any menu from the **menu bar** (top) to open a panel.

The most important panels are **Acquire**, **Image Display (4)** and **Control Panel (3)** (global).



The **CONTROL PANEL** (3) allows controlling the light intensity of both the fluorescence lamp and the lasers.

NOTE: Do not oversaturate your image. Saturation can be displayed in any wished Pseudocolor by checking the Box "Show Saturation" (4, see Image Display below).



The **IMAGE DISPLAY** panel (**4**) can be opened either in the **menu bar** or by clicking with the right mouse button on any image. Normally, **Auto Scale** (**A**) is tagged by default, which corrects each and every frame according to the selected range (**B**). If not needed, deselect the Auto Scale box. Use the sliders below (**C**) to offset the intensity and background. Any of the settings established can be used as default for acquisition if the **"Use as Acquire Default"** button (**D**) is clicked.

The pseudo display color of your images of each channel can also be changed in this panel.

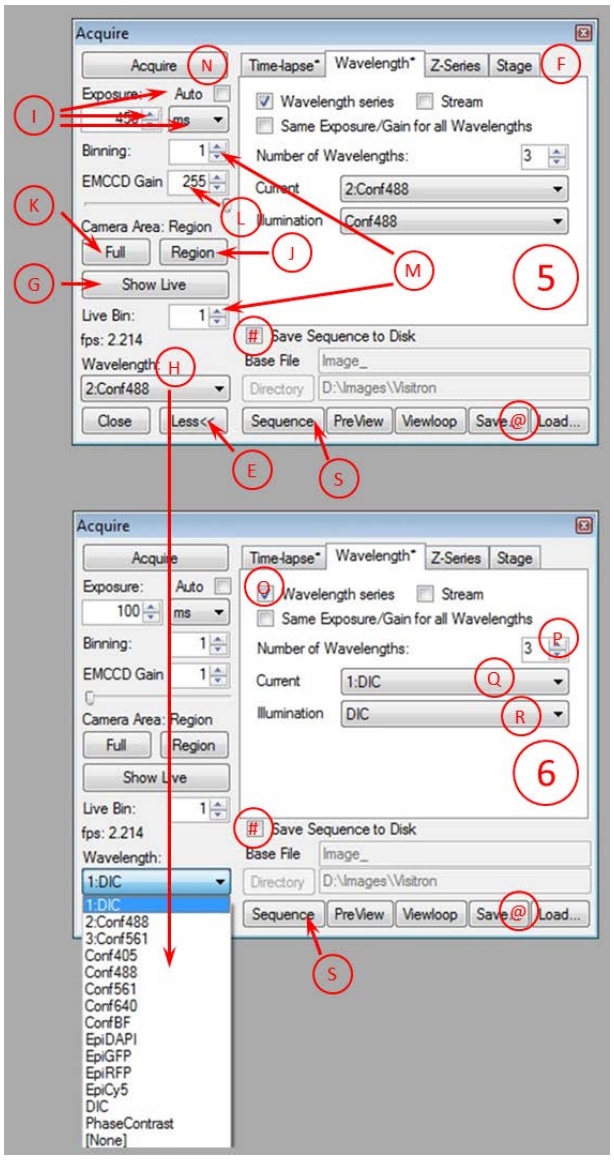
If the "**X Y Scale Bar**" is tagged, it needs to be stamped to be saved with the image.

A **Timestamp** for time lapse images can be also implemented in the acquired images.

The **AQUIRE PANEL** (**5, 6, 7 and 8, see also below**) can be maximized (multidimensional imaging) or simplified if you click the **"more/less"** button (**E**). If it is maximized, the four tabs (**F**) to control acquisition parameters –Time-lapse, Wavelength, Z-series and Stage– are visible. See detailed explanations below.

The * on each tab shows which one is activated.

Click on **"Show Live"** (**G**) to change the light path to the camera and visualize your specimen in the screen. You might also have to change the light path manually in the microscope (press button **F16 on the microscope**). Choose the channel you want to see in the **wavelength drop down menu** (**H**). Keep in mind that for bright field imaging, the 20 x air and 40 x air objectives support **Phase Contrast,** but not **DIC** and it is the other way around for the rest of the objectives.



NOTE: for **DIC** the cooled LED is used. Always take into account to also play around with its intensity beside changing exposure time and gains etc.

NOTE: for **Phase Contrast**, the objective condenser aperture needs to be fully open.

For every channel, the **exposure time** (**I**) can be set either automatically or manually. But, auto exposure is NOT recommended.

NOTE: The exposure time should not be below 100 ms, when using the full area of the camera, because otherwise the camera and the disc are not synchronized and banding of the image might appear.

NOTE: for exposure times stick to the 5ms pattern – e.g. 100, 95ms but not 112

If you are interested in only a smaller part of the field, the exposure time may be decreased below 100 ms without banding.

To zoom in a R**egion Of Interest (ROI)**, click on **"Region"** (**J**), define the place you want to focus with the mouse and click **"Region"** again. To go back to the general picture, click **"Full"** (**K**).

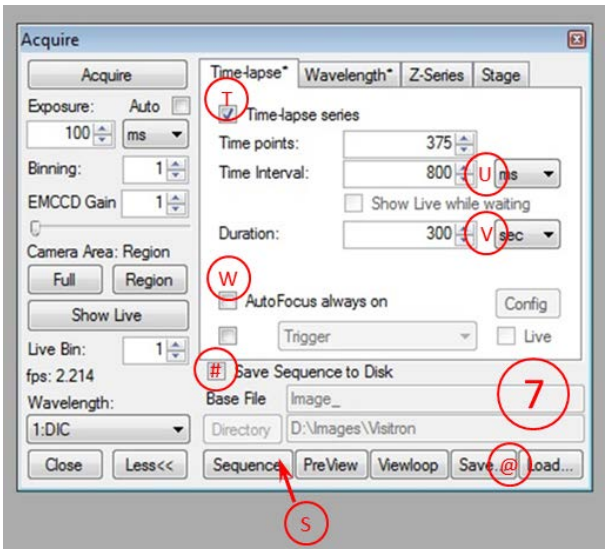
For deleting the ROI(s) use the macro on the right panel.

The **EMCCD gain (L)** is a non-linearized gain, it is exponential. It does not add additional noise to the image. The increase in SNR (signal to noise ratio) gets higher towards the end → a gain between 100 and 180 may be the best value.

By **hardware/pixel binning** (**M**) the spatial resolution gets decreased substantially, while the SNR increases and the experiment get faster. Pixel binning is a clocking scheme used to combine the charge collected by several adjacent CCD pixels, and is designed to reduce noise and improve the signal-to-noise ratio (SNR) and frame rate of digital cameras. Increase the value to increase the number of pixels combined. **Live binning** may be used during focusing to minimize photo bleaching, as the exposure time and laser power can be decreased.

Once everything is set, click on **"Acquire"** (**N**) to take a snapshot. This takes a single frame in the active channel and can be saved as a .tiff file.

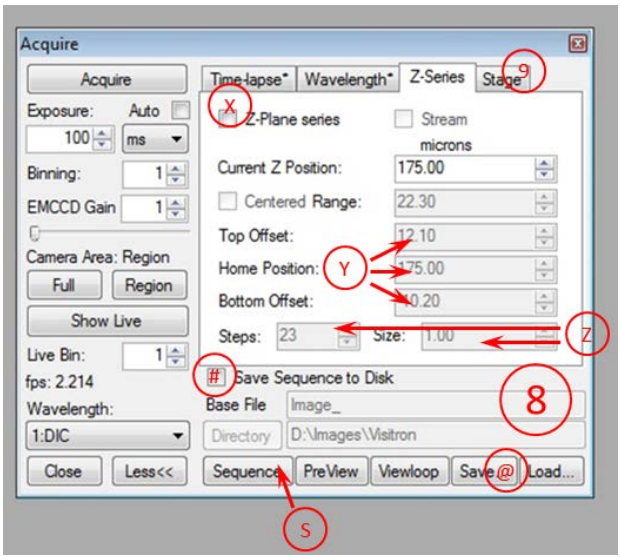
To activate/inactivate a multichannel image, go to the **WAVELENGTH** tab (**6**) and tick/untick the **"Wavelength series"** box (**O**). Set the number of channels you want to have active during acquisition (**P**). This limits the number of available choices in the **"Current"** scroll bar (**Q**). Go one by one choosing the laser in the **"Illumination"** scroll bar (**R**). For each channel it can be defined if a z-stack will be taken or not. To take a snapshot in all the chosen channels, click on **"Sequence"** (**S**).



To take a time-lapse video, go to the **TIME-LAPSE** tab (**7**) and tick the **"Time-lapse series"** box (**T**). Define the time interval between each frame (**U**) and the duration of the acquisition (**V**). Choose the time interval according to your needs, i.e. speed of movement/biological event, but take into account that it will readjust if you don't consider the exposure time and the number of planes (minimal time interval = exposure time of all your channels * number of Z planes). Tick the box **"AutoFocus**

always on" (**W**) to engage the PFS while acquiring and to avoid focus drift. Click on **"Sequence"** (**S**) once you want to start acquisition.

After the time lapse is taken, you can toggle through the different time points by using the arrows ← →.



To take a Z-stack, go to the **Z-SERIES** tab (**8**) and click the **"Z-Plane series"** box (**X**). You can either define a plane of interest manually with the z-focus wheel to set the **"Home position"** and then set different **"Top-"** and **"Bottom Offset"** (**Y**) or set in a region of interest the number of planes under **"Steps"** and the Z-size of your planes under **"Size"** (**Z**). The number of planes will change automatically when you modify **"Top-"** and **"Bottom Offset"** parameters. These can be modified either with the little arrows next to the box or if you click in the box and scroll with the mouse. In the wavelength tab you can define if a z-stack should be taken for which wavelength. The image will be taken in the home position of the channels there no z-stack is defined. Click on **"Sequence"** (**S**) once you want to start acquisition.

To toggle between the z-positions in the acquired image use the arrows up and down.

In the **STAGE** tab (**9**), you can control not only the Z plane but also the X,Y position. Here different positions can be defined (same z-stack size will be applied, but is relative to the home position). If you tick the box "relative", all positions will be moved in relation to the changed one. In the stage tab a tile scan option can be also found.

To toggle between the different positions in the acquired image use the arrows up and down.

Finally, any special settings in the multidimensional acquisition settings you may use frequently can be saved and loaded any time later (**@**).

SAVING YOUR DATA

If the **“Save Sequence to Disk”** is unchecked during an acquisition sequence or if individual shots have been taken using the **“Acquire” (N)** button the images are saved to ram and must be saved to disk manually using the file menu. Both time-lapse videos and stacks can be saved as **.stk** (multi-slice .tiff) files. Any software like ImageJ and Fiji should open these files without any need of a special plug-in.

You may take Z-Series and Time-lapse videos at the same time but beyond a certain complexity sequences must be saved directly to a directory on the disk (The **“Save Sequence to Disk”** (**#**) option cannot be unchecked). This generates big sequences of images and an **.nd** file which summarizes all data in an acquisition sequence. The .nd file can be used to open all files in a sequence simultaneously with VisiView or other programs. In ImageJ/Fiji the opening of .nd files requires a plug-in (nd stack builder). In general it’s recommended to always let the software save your sequences automatically to disk: tick the box **“Save Sequence to Disk”** (**#**) in the **Acquire** panel and choose a directory and the default name of your pictures under **“Base File”**.